Effect of the Nematode Aphelenchus avenae on Some Ectotrophic, Mycorrhizal Fungi and on a Red Pine Mycorrhizal Relationship

Jack R. Sutherland and J. André Fortin

Forest Research Laboratory, Department of Forestry and Rural Development, and Faculté de Forestière et Géodésie, Université Laval, Québec, Canada. Present address of senior author: Forest Research Laboratory, Victoria, British Columbia, Canada.

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ABSTRACT

The mycophagous nematode *Aphelenchus avenae* fed and reproduced on seven species of ectotrophic, mycorrhizal fungi grown in vitro. The best host for nematode reproduction was *Amanita rubescens*, followed by *Suillus punctipes, S. granulatus, S. luteus, Russula emetica, Cenococcum graniforme,* and *Rhizopogon roseolus*. The latter apparently produced a substance which was toxic to the nematode. Inoculum levels of 5, 10, 25, and 50 nematodes/plate retarded the growth of all the mycorrhizal fungi. Reduction in growth of the fungi was related more to the number of nematodes in the inoculum than to the final nematode populations in the plates. The nematode's mode of feeding and the visible effects of feeding on the fungi are described.

In pure culture, *A. avenae* prevented the formation of an ectotrophic, mycorrhizal relationship between red pine (*Pinus resinosa*) and *Suillus granulatus*. Several possible implications of mycorrhizal suppression by mycophagous nematodes are discussed.

RÉSUMÉ

Sept champignons mycorhizateurs ectotrophiques ont supporté, in vitro, la nutrition et la reproduction d'*Aphelenchus avenae*. Le meilleur hôte fut *Amanita rubescens*, suivi de *Suillus punctipes, S. granulatus, S. luteus, Russula emetica, Cenococcum graniforme,* et *Rhizopogon roseolus*. Il semble que ce dernier champignon produise une substance toxique pour le nématode. En utilisant différentes concentrations d'inoculum (0, 5, 10, 25, et 50 nématodes/plat) il s'est avéré que l'effet néfaste exercé par *A. avenae* sur la croissance des champignons était en relation plus directe avec le nombre de nématodes présents dans l'inoculum qu'avec le nombre final de ceux-ci. La façon selon laquelle le nématode se nourrit du mycélium des différents champignons ainsi que ses effets sur ceux-ci sont décrits.

Il fut démontré, en cultures pures, que *A. avenae* peut empêcher le champignon *S. granulatus* de former des mycorhizes avec le pin rouge (*Pinus resinosa*). La suppression des mycorhizes, par les nématodes mycophages, implique diverses conséquences possibles qui sont discutées ici.

The nematode *Aphelenchus avenae* Bastian, 1865, is commonly found in the rhizosphere of plants. Steiner (9) claimed that *A. avenae* was parasitic on higher plants, but the current concept is that *A. avenae* is primarily mycophagous (2) and that in certain cases (11) it may feed on the cells of higher plants. *A. avenae* is prevalent in most forest nursery soils, and it has been suggested (10, 12) that by feeding on mycorrhizal fungi it could prevent or partially inhibit the formation of the mycorrhizal relationship with tree seedlings. This paper reports on: (i) the success of *A. avenae* in feeding and reproducing on seven species of mycorrhizal fungi growing in vitro; (ii) the type and amount of damage done to the fungi by the feeding activities of the nematode; and (iii) the effect of nematode feeding on the establishment of an ectotrophic, mycorrhizal relationship between red pine (*Pinus resinosa* Ait.) seedlings and *Suillus granulatus* (L. ex Fr.) Kuntze.

MATERIALS AND METHODS.—*Sources of inocula.*—The mycorrhizal fungi used in this study were *Amanita rubescens* (Fr.) S.F.Gray, *Cenococcum graniforme* (Sow.) Ferd. & Winge, *Rhizopogon roseolus* (Cda.) Th. Fr., *Russula emetica* (Fr.) S.F.Gray, *Suillus granulatus, S. luteus* (Fr.) S.F.Gray, and *S. punctipes* (Peck) Sing. All except *S. granulatus* were subcultures of the isolates of Hacskaylo et al. (3). *Suillus granulatus* was isolated in 1965 from a young sporophore in a spruce stand at Duchesnay, Quebec. Stock cultures were maintained at 4 C on glucose-ammonium tartrate agar composed of 10 g of glucose, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of ammonium tartrate, 2.5 ml of ferric citrate-citric acid solution (1% of each), 0.5 ml of 1% $ZnSO_4 \cdot 7H_2O$ solution, 10 g of malt extract, 15 g of agar, and distilled water to make 1 liter of medium. This medium is a modification of that of Melin and Das Rama (7). Before autoclaving, the pH of the medium was adjusted to 4.5 with HCl. Fungi for inoculum were cultured at 25 C on this medium in petri dishes.

The origin of the *A. avenae* population and methods for obtaining aseptic nematode inoculum are given elsewhere (10). Nematode inoculum was extracted and used when 10-14 days old.

Influence of A. avenae on the growth of the mycorrhizal fungi in vitro, and reproduction of the nematode.—Cultures of each fungus were grown in 9-cm petri dishes by center-inoculating the glucose-ammonium tartrate agar medium with 0.5-cm plugs, mycelium down, cut from 2- to 3-week-old cultures. They were grown for 24 hr before the nematodes were added at rates of 0, 5, 10, 25, or 50/plate. The nematodes in 0.1-0.3 ml of sterile, distilled water were pipetted onto the fungus inoculum block. The control (0 nematode inoculum level) cultures received only sterile, distilled water because Seitz-filtered water which had contained nematodes had no effect on growth of the fungi. Each nematode inoculum level-fungus combination was replicated six times. The cultures were randomized and incubated at 25 C in polyethylene bags. After 3 weeks

the diameters of the colonies were measured and the nematodes extracted using a modified Baermann funnel technique. Extraction time was 48 hr, after which 10 ml of water were taken from each funnel and the nematodes counted using a stereomicroscope. Dilutions and appropriate conversion factors were used to determine the nematode populations. The means were compared using Duncan's multiple range test (8).

Nematode feeding and its effect on the fungi.—Nematode activity, feeding, and the type of damage done to the mycelia were observed by placing thin agar blocks from nematode-fungus cultures on microscope slides. A cover slip was added and nematode activity observed with a compound microscope.

Influence of A. avenae on the formation of an ectotrophic, mycorrhizal relationship on red pine.—Erlenmeyer flasks (500 ml) were cleaned with chromic acid solution, rinsed in distilled water, and dried. Each flask received 250 cm^3 of screened vermiculite (about 1 cm in diam) and 125 ml of medium composed of 2.5 g of glucose, 250 mg of $(NH_4)HPO_4$, 50 mg of $CaCl_2$, 25 mg of NaCl, 500 mg of KH_2PO_4, 150 mg of $MgSO_4 \cdot 7H_2O$, 3 ml of ferric citrate-citric acid solution (1% of each), 25 μg of thiamine, and 1,000 ml of distilled water. The pH of the medium was initially adjusted to 4.5 with HCl to achieve a final pH of 6.4-6.7 after addition of the vermiculite (6). The tops of the flasks were plugged with gauze, capped with aluminum foil, and the flasks autoclaved for 20 min at 121 C.

Red pine seeds were surface sterilized with 1:1,000 aqueous $HgCl_2$, germinated aseptically on glucose-yeast extract agar medium (11), and used when the radicles had just started to emerge. Inocula of *S. granulatus* and of *A. avenae* were obtained as described earlier. The treatments were: (i) red pine, (ii) red pine plus *S. granulatus*, and (iii) red pine plus *S. granulatus* plus 25 *A. avenae*. There was no treatment of red pine plus *A. avenae* because previous studies (10) showed that the nematode does not feed on red pine under aseptic conditions. A depression (about 1.5 cm deep and 3.5 cm in diam) was scooped with a sterile inoculation needle in the vermiculite of each flask. One red pine seed was placed in the middle of each depression, and three pieces of fungus inoculum (1-cm-diam agar blocks) were placed around it at a distance of about 1 cm. Twenty-five nematodes in 5 ml of sterile, distilled water were pipetted onto and around the seed and fungus. Control flasks received only 5 ml of sterile, distilled water. Each treatment was replicated 20 times. After inoculation, the flasks were placed randomly in a growth chamber at 23 C and 3,000 ft-c of light for 18 hr and 17 C with no light for 6 hr. Relative humidity was kept at 65%.

The experiment was evaluated 5 weeks after inoculation. Tests for contaminants were made by transferring vermiculite from the Erlenmeyer flasks to test tubes containing liquid glucose-yeast extract (for bacteria) and glucose-ammonium tartrate agar (for fungi).

Seedlings were removed from the flasks, the roots cleaned of adhering vermiculite, and placed on moist filter papers in petri dishes. Using a stereomicroscope, the number of mycorrhizal points (any short branch of a long, lateral root was counted as one mycorrhizal point regardless of its number of dichotomous branches) on each root system was determined, and the cumulative length of those sections of the seedling root system on which mycorrhiza were present was calculated.

Two techniques were used to determine if *A. avenae* had entered the roots: (i) Six root systems, randomly selected, were incubated for 2 weeks in 30 ml of distilled water; (ii) three other randomly selected root systems were washed with a spray of water, stained with acid fuchsin, cleared with lactophenol (13), dissected, and examined using a stereomicroscope. The tops and roots of the seedlings were severed at the root collar, dried overnight at 80 C, cooled for 1 hr in a $CaCl_2$-charged desiccator, and weighed. The nematodes in the vermiculite in each flask were extracted using a modified Baermann funnel technique. Nematode counts were made as for the plate cultures. Because some of the flask cultures became contaminated, and some root systems were used for nematode examination, resulting in unequal numbers of replicates, the results were subjected to special analysis of variance (8) and Duncan's multiple range test. Since the validity of the latter test has not yet been verified (8), comparisons between adjacent means were made with a t-test for unequal replicates.

RESULTS.—*Influence of A. avenae on the growth of the mycorrhizal fungi in vitro, and reproduction of the nematode.*—*Aphelenchus avenae* reproduced on all seven of the mycorrhizal fungi and caused a reduction in their growth (Table 1). The best fungus host for the nematode was *A. rubescens*, followed by *Suillus* spp., *R. emetica*, *C. graniforme*, and *R. roseolus*. In most cases, the amount of damage (reduction in growth)

TABLE 1. Influence of *Aphelenchus avenae* on the growth[a] of seven ectotrophic, mycorrhizal fungi

Initial no. of nematodes	*Amanita rubescens*		*Cenococcum graniforme*		*Rhizopogon roseolus*		*Russula emetica*		*Suillus granulatus*		*Suillus luteus*		*Suillus punctipes*	
	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes	Diam of fungus colony	Final no. of nematodes
	mm	*no.*	*mm*	*no.*	*mm*	*no.*	*mm*	*no.*	*mm*	*no.*	*mm*	*no.*	*mm*	*no.*
0 (control)	49 a[b]	0 a	22 a	0 a	90 a	0 a	35 a	0 a	40 a	0 a	49 a	0 a	34 a	0 a
5	47 a	18,166 b	16 b	6,866 b	90 a	50 b	26 b	26,122 b	34 b	5,322 b	46 a	300 b	28 b	8,077 b
10	43 a	57,055 c	15 b	6,733 b	90 a	37 b	21 b	23,555 b	26 c	18,011 bc	48 a	733 b	20 c	42,089 d
25	44 b	57,555 c	14 bc	10,900 b	88 ab	136 bc	19 c	33,150 b	21 d	34,811 cd	44 a	6,445 b	16 cd	37,533 cd
50	25 c	81,360 d	11 c	15,022 c	84 b	217 c	12 d	31,589 b	16 e	37,011 d	28 b	31,522 c	13 d	26,711 c

[a] Numbers are means of six replicate cultures, 5 weeks after nematodes were added.

[b] Reading down the columns, numbers followed by a letter in common are not significantly different at the .01 level.

was more dependent upon the number of nematodes in the inoculum than upon the final number of nematodes in the plates. Usually, the more suitable the fungus was as a host, the more it was damaged by the nematodes. The graduated nematode inoculum levels did not always produce corresponding gradations in final nematode populations.

The most noticeable macroscopic effect (besides the smaller diameter of the colonies) of the nematode on all the fungi, except *R. roseolus,* was the suppressed, watery appearance of aerial mycelium. When many nematodes were present, the growth of the fungi that would normally be on the surface of the agar was submerged. *Rhizopogon roseolus* was the only fungus not visibly damaged by the nematode. The fungi were not killed by the nematode because mycelium grew from inoculum blocks cut from the margins of 3-week-old colonies of each nematode inoculum level.

Nematode feeding and its effect on the fungi.—The seven mycorrhizal fungi tested can be placed in three categories, based upon the type of nematode feeding and host response. The first category includes *A. rubescens, R. emetica, S. granulatus, S. luteus,* and *S. punctipes.* On these fungi, adult and larval *A. avenae* feed at random on all ages of mycelial growth, i.e., on older hyphae (nearest the inoculum block) and on young hyphal tips at the periphery of the fungus colonies. Only two to four stylet jabs were required for the nematode to make a feeding wound in a hypha. During feeding, hyphal cytoplasm was rapidly pulled from both directions on each side of the wound toward the tip of the nematode's stylet. The nematode's median bulb valve pulsated rapidly during feeding. Removal of the cytoplasm caused the hyphal walls to collapse for some distance on each side of the wound. The time from initiation of wounding to stylet withdrawal did not exceed 5 sec. After feeding, the nematode moved and fed on the next hypha or another part of the same hypha. Attempts to feed on collapsed hyphae were unsuccessful.

Feeding on *C. graniforme* differed from that on the above fungi. Most of the nematodes remained near the edge of the fungus colony where they fed mainly on or near the tips of slender hyphae (Fig. 1). Five to 10 stylet jabs were required to make a feeding wound. In contrast to the feeding activity on the above fungi, *A. avenae* fed for a longer time at one place on *C. graniforme,* and the time between pulsations of the median bulb valve was much longer. Searching time, i.e., the interval between cessation of feeding at one site and initiation of wounding at the next, was much longer due to the nematodes' preference for hyphal tips. Removal of hyphal cytoplasm by nematode feeding caused the walls to collapse and the tips of the hyphae to shrivel (Fig. 2, 3).

Rhizopogon roseolus was the only fungus tested on which *A. avenae* did not reproduce well. This is attributed to a toxic factor produced by young mycelium of the fungus. Nematodes that were placed on cultures growing on the glucose-ammonium tartrate agar medium did not feed on the fungus. The nematodes were active for about 3 days, whereupon the majority became quiescent. When active, they frequently "nudged" hyphae but did not feed. The esophagous and intestine of dying nematodes gradually became brownish orange and shriveled upon death. There was no indication that the nematodes were trapped or infected by fungus mycelium. Eggs that were laid developed and the larvae emerged. Apparently, the fungus produces the toxin only when proper nutrients are in the medium because the nematode feeds and some reproduction occurs on mycelium growing from agar inoculum blocks on 2% water agar. It seems that only the youngest (nearest the periphery of the colony) mycelium produced the toxin. This might explain the slight amount of *A. avenae* reproduction on the fungus (Table 1). Nematodes that did reproduce were thought to do so by confining their activities to older mycelium, thus escaping contact with the toxin. This hypothesis was tested by using the flood-plate technique (4) to obtain even-age mycelium over the entire surface of glucose-ammonium tartrate agar medium in petri dishes. The inoculum was grown at 25 C on 25 ml of liquid glucose-ammonium tartrate medium in 250-ml Erlenmeyer flasks for 1 month. Mycelium from four of these cultures was combined and cut for 25 sec in a Waring Blendor with 50 ml of distilled water. The surface of the agar was flooded with 1 ml of cut mycelium. Twenty-five *A. avenae* were added to each plate at intervals of 1, 2, 3, and 5 weeks after the fungus was started. Each treatment was replicated three times. One month after the nematodes had been added, they were extracted and counted. The mean numbers of *A. avenae* per plate were 0, 1,057, 1,733, and 7,409 for the fungus cultures that had received nematodes at 1, 2, 3, and 5 weeks, respectively. These results indicate that the toxin is produced by young hyphae of *R. roseolus.*

Influence of A. avenae on the formation of an ectotrophic, mycorrhizal relationship on red pine.—The results of this experiment are given in Table 2. By feeding on the fungus component of the relationship, *A. avenae* caused a significant reduction in the abundance of mycorrhizae on the red pine root systems. The number of mycorrhizal points and the cumulative lengths of root sections with mycorrhizae (two different ways of expressing the intensity of a mycorrhizal relationship) were both less when the nematode was present (Fig. 4, 5). The range for the number of mycorrhizal points on seedlings where nematodes were present was 0-44, and 15-58 where they were absent. Although a statistical analysis was not made, it was evident that the final nematode population had to exceed 10,000/flask before a reduction in the number of mycorrhizae occurred. The nematodes did not kill the fungus because *S. granulatus* grew on the media used to check for bacterial and fungal contaminants. Microscopic examination of sections, cut with a freezing microtome, from several mycorrhizae showed that the nematodes had not altered the morphology of the mycorrhizae (Fig. 6). The nematodes did not affect seedling top or root weight.

Aphelenchus avenae was not obtained from the incubated roots, nor observed within or upon the tissues of stained roots.

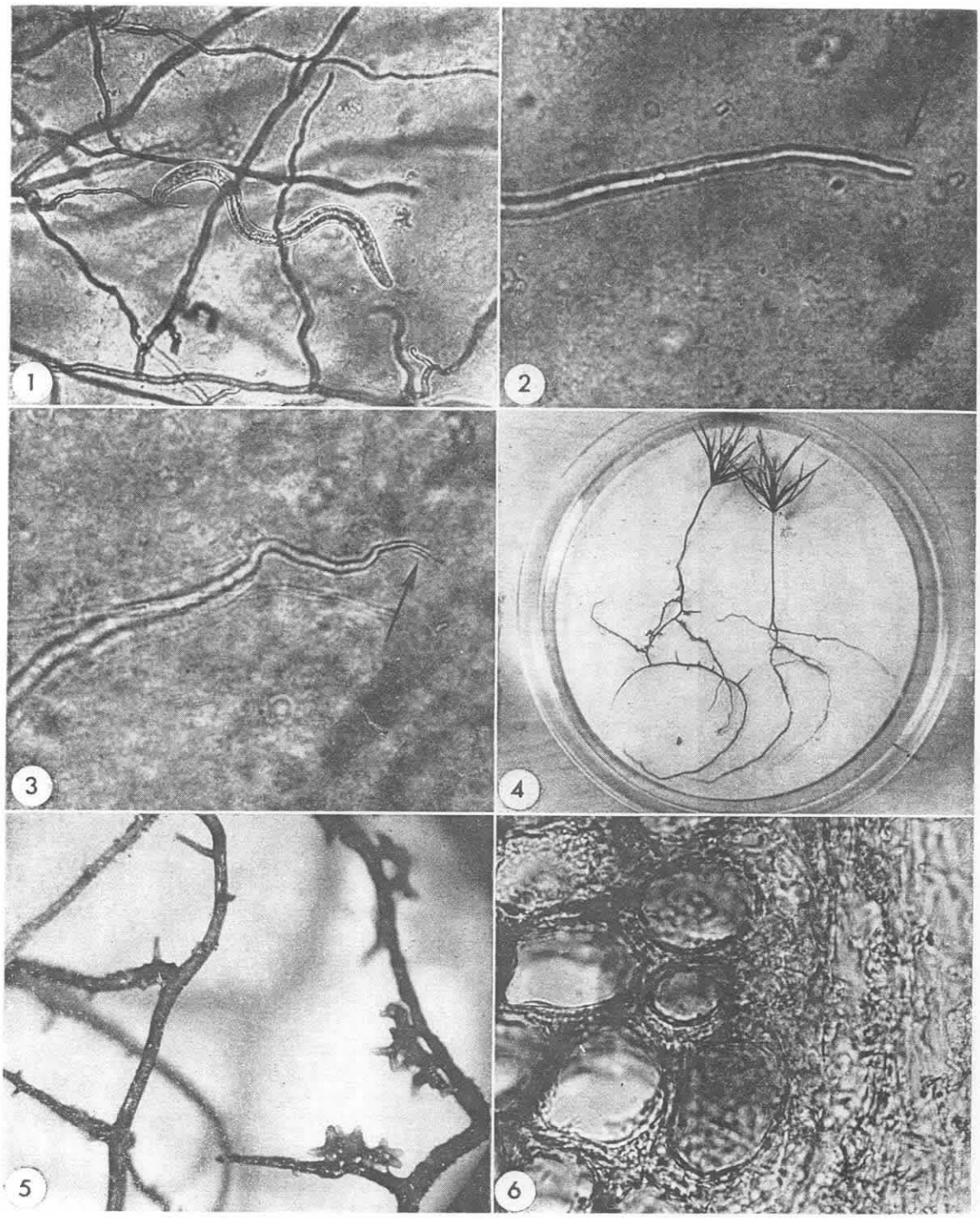

Fig. 1-6. Effect of the nematode *Aphelenchus avenae* on the mycorrhizal fungus *Cenococcum graniforme*, and on a mycorrhizal relationship between red pine and *Suillus granulatus*. **1)** *A. avenae* feeding on tip of a *C. graniforme* hypha. **2)** Hyphal tip before nematode fed. **3)** Shriveled hyphal tip after nematode fed. **4)** Mycorrhizae formed on seedling at left in treatment without nematodes; seedling at right from flask with nematodes. **5)** Enlargement showing effect of *A. avenae* on mycorrhizal formation; roots at right from flask without nematodes. **6)** Cross section of a red pine mycorrhiza with well-developed Hartig net showing that mycorrhizae which did form when the nematode was present were morphologically normal.

TABLE 2. Influence of *Aphelenchus avenae* on an ectotrophic, mycorrhizal relationship between *Suillus granulatus* and red pine[a]

Treatment	Dry wt		Mycorrhizal points	Mycorrhizal root length	Nematodes/flask	
	Shoots	Roots			Vermiculite	Roots
	mg	*mg*	*no.*	*mm*	*no.*	*no.*
Red pine[b]	23.2 a	7.3 a	0 a	0 a	—	—
Red pine plus *S. granulatus*[c]	24.7 a	13.3 b	35.8 b	68.4 b	—	—
Red pine plus *S. granulatus* plus 25 *A. avenae*[d]	26.2 a	15.9 b	16.2 c	31.9 c	15,543	0

[a] Means followed by the same letter in a column are not significantly different at the .01 level.
[b] Means based on 19 replicates.
[c] Means based on 16 replicates.
[d] Means based on 19, 10, 19, 19, 19, and 9 replicates (reading across), respectively.

DISCUSSION.—Although *A. avenae* has been propagated on a wide variety of saprophytic and plant-pathogenic soil fungi (5, 14), this is the first report of the nematode feeding and reproducing on mycorrhizal fungi and retarding fungal growth. The feeding activities of the nematode can suppress the formation of a specific mycorrhizal association of red pine. This type of relationship between mycophagous nematodes and mycorrhizae will probably prove to be widespread. Mycophagous nematodes may be indirectly more important to plant growth and disease development than has been suspected. For example, by retarding the development or preventing the formation of mycorrhizae, fungus-feeding nematodes could indirectly affect the development of plants that require the relationship to maintain optimum growth. Nematode feeding activities might also impair the efficiency of established mycorrhizae as absorbing structures, or predispose them to destruction by other soil microorganisms. The activities of mycophagous nematodes could have implications not yet recognized with regard to root diseases caused by pathogenic fungi. Zak (15) gives several ways in which mycorrhizal fungi might protect roots from attack by damping-off and root rot fungi. By suppressing mycorrhizal formation, mycophagous nematodes such as *A. avenae* could indirectly contribute to the development of these diseases, especially when the pathogens are poor hosts or nonhosts [e.g., some species of *Pythium* (5, 14) and *Phytophthora* (14)] of certain mycophagous nematodes. As forest nursery seedbeds are often rapidly recolonized by large numbers of mycophagous and bacterium-feeding nematodes following soil fumigation (12), some or all of the above situations may occur.

The finding that *A. avenae* does not enter roots indicates that the nematode cannot destroy established mycorrhizae. The nematode probably suppresses mycorrhizal formation by preventing the fungus from reaching the root symbiont of the relationship or by so weakening the fungus that even if it does come into contact with the root, it is unable to enter into mycorrhizal relationship. The failure to find *A. avenae* in either root tissues or mycorrhizae further suggests that this nematode cannot enter roots unless preceded by a pathogenic fungus. This observation strengthens the hypothesis that *A. avenae* cannot enter or feed upon intact, healthy tissues of higher plants.

The apparent ability of *R. roseolus* to produce a substance toxic to *A. avenae* deserves further consideration because this fungus, or others, might be used to synthesize mycorrhizae.

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